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Development and Cytomolecular Identification of Monosomic Alien Addition and Substitution Lines of Triticale (\times *Triticosecale* Wittmack) With 2S^k Chromosome Conferring Leaf Rust Resistance Derived From *Aegilops kotschy* Boiss

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Alien chromosome introgression has become a valuable tool to broaden the genetic variability of crop plants via chromosome engineering. This study details the procedure to obtain monosomic addition and monosomic substitution lines of the triticale carrying 2S^k chromosome from *Aegilops kotschy* Boiss., which harbors *Lr54* + *Yr37* leaf and stripe rust-resistant gene loci, respectively. Initially, *A. kotschy* \times *Secale cereale* artificial amphiploids ($2n = 6x = 42$ chromosomes, UUSSRR) were crossed with triticale cv. “Sekundo” ($2n = 6x = 42$, AABRRR) in order to obtain fertile offspring. Cytomolecular analyses of five subsequent backcrossing generations revealed that 2S^k chromosome was preferentially transmitted. This allowed for the selection of monosomic 2S^k addition (MA2S^k) lines of triticale. Finally, the 2S^k(2R) substitution plants were obtained by crossing MA2S^k with the nullisomic (N2R) plants of triticale. The presence of 2S^k chromosome in subsequent generations of plants was evaluated using SSR markers linked to *Lr54* + *Yr37* loci. Disease evaluation of the monosomic 2S^k(2R) substitution plants for the reaction to leaf and stripe rust infection were carried out under controlled conditions in a growth chamber. The results showed significant improvement of leaf rust resistance severity of monosomic substitution plants compared with control (“Sekundo”). In contrast, the introgression of the *Lr54* + *Yr37* loci did not lead to improvement of stripe rust resistance. In summary, the creation of monosomic addition and monosomic substitution lines of triticale is the starting point for the precise and guided transfer of *Lr54* + *Yr37* loci. The results showed that the developed materials could be exploited for the development of triticale varieties with resistance to leaf rust.

Keywords: back-cross, fluorescence in situ hybridization, molecular markers, monosomic, resistance genes, triticale 2, *Aegilops kotschy*

INTRODUCTION

Wild relatives and related species with homoeologous genomes are important for broadening the genetic variability of crop plants. For many crops, wild relatives provide a vast reservoir for most of agronomically important traits. A large number of the highest yielding wheat (*Triticum aestivum* L.) cultivars carry portions of alien chromatin introgression from related crops (*Secale cereale* L.) or weedy species (*Aegilops* sp., *Dasyphyrum* sp., *Elymus* sp., *Haynaldia* sp.) (Gill et al., 2011). The *Aegilops* genus is the closest wild relative of bread wheat (*T. aestivum*; $2n = 6x = 42$ chromosomes; AABBDD) or triticale (\times *Triticosecale* Wittmack; $2n = 6x = 42$; AABBRR). It contains 11 diploid, 10 tetraploid, and 2 hexaploid species and provides a vast reservoir of valuable genes, which were eliminated during the domestication and breeding of cultivated cereals.

Aegilops kotschy Boiss. ($U^kU^kS^kS^k$, $2n = 4x = 28$) is valuable from genetic and breeding point of view as it carries genes associated with disease resistance, drought, heat, salt tolerance, as well as other beneficial traits (Schneider et al., 2008). Rawat et al. (2009) utilized *A. kotschy* as a source of genes to increase the iron and zinc content in seeds of wheat. *A. kotschy* was crossed as a male parent with bread wheat line “Chinese Spring” lacking the *Ph1* locus, which is a suppressor of homeologous pairing in wheat (Riley and Chapman, 1958). The aim of their study was to bring the genes associated with increased iron and zinc content in seeds, harbored in the $2S^k$ and $7U^k$ chromosomes of *A. kotschy*, into the genome of wheat. They obtained only a $2S^k$ and $7U^k$ chromosome substitution line, which were not very suitable for commercial exploitation due to linkage drag. It is also reported that chromosome $2S^k$ of *A. kotschy* possess leaf and stripe rust-resistant loci of *Lr54* and *Yr37* genes (Marais et al., 2005; Prazak and Paczos-Grzęda, 2013). Marais et al. (2005) have introduced *A. kotschy*-derived leaf and stripe rust-resistant genes *Lr54* and *Yr37* to the wheat genome by the induction of $2DS.2S^kL$ chromosome translocation.

Polyploids, such as of cultivated forms wheat or triticale, contain multiple sets of chromosomes and have highly buffered genotypes that are more permissive to benefit from alien introgression than diploids (Qi et al., 2007). Monosomic alien addition lines (MAALs) contain only one alien chromosome in addition to the receptor background chromosomes. The production of monosomic addition lines rely on backcrosses with an acceptor crop (Faris et al., 2002; Brar and Dhaliwal, 2004; Wulff and Moscou, 2014; Kwiatek and Nawracała, 2018). In traditional breeding of intraspecific hybrids, the recurrent backcrossing is commonly employed to transfer alleles at one or more loci from a donor to an elite variety (Veatch-Blohm, 2007). The expected recurrent parent genome recovery would be 99.2% at the end of six backcrossing, which is most similar to improved variety. The proportion of the recurrent parent genome is recovered at a rate of $1 - (1/2)^t + 1$ for each of the generations of backcrossing (Singh and Singh, 2015). When generating interspecific hybrids, backcrossing is required to generate addition, substitution, and translocation lines. In wheat and triticale, chromosome pairing between homologous chromosomes is strictly regulated by the expression of *Ph1*

and *Ph2* loci (Riley and Chapman, 1958; Sears, 1977). Thus, subsequent backcrosses eliminate the alien chromatin gradually. However, the alien chromosome can be transmitted to the offspring preferentially, which contributes to the saturation of specific chromosomes (Endo, 2007).

Some *Aegilops* chromosomes were reported to be transmitted preferentially to the offspring in the process of backcrossing to produce alien chromosome addition lines of wheat (Endo, 2007) and triticale (Kwiatk et al., 2016c). The gametocidal activity of genes located on chromosome $4S^{sh}$ from *A. sharonensis* Eig is an example of when a chromosome is preferentially eliminated (Endo, 1990). Such chromosomes were reported to carry gametocidal factors (*Gcs*) (Endo, 1985). These chromosomes are known to remain in host plants in a “selfish” way. The preferential transmission of *Gc* chromosomes result from the elimination of gametes that lack them, while the gametes with the *Gc* chromosomes remain fertile (Nasuda et al., 1998). *Gc* factors cause extensive chromosome breakage, which results in the induction of non-functional gametes and exclusive transmission of the *Gc* chromosome to the offspring (Nasuda et al., 1998). *Gcs* have been reported in *A. geniculata*, *A. triuncialis*, *A. caudata*, *A. cylindrica*, *A. longissima*, *A. sharonensis*, and *A. speltoides*. Their activity is mostly confined to 2, 3, and 4 homeologous groups of C, S, S^1 , S^{sh} , and M^g genomes. It has been reported that *Gc* genes can be constructively utilized for development of addition, deletion and translocation stocks in wheat (Endo, 2007) and triticale (Kwiatk et al., 2017b).

Monosomic alien addition and substitution lines (MAALs and MASLs) are widely used as linking bridges for the transfer of desirable genes from wild species into elite cultivars. For example, wheat-rye addition, substitution, and translocation lines are used as pre-breeding materials for the improvement of wheat (Lukaszewski, 2000, 2016) and triticale (Lukaszewski, 2006). MASLs can be used to identify favorable genes in wild species, allowing for more accurate and rapid transfer of such genes to create introgression lines. Such initiatives provide the opportunity to examine the effect of specific alien (Edet et al., 2018) and to construct the physical maps of specific chromosomes (Kynast et al., 2001). MASLs are produced by crossing monosomic or nullisomic acceptors with appropriate addition lines (Sears, 1972). The missing chromosome of acceptor plant is substituted by the donor homoeologue. The production of substitution lines for alien chromosomes is the initial step for direct introgression of specific alien chromosome segments into the acceptor genome (Lukaszewski, 2000, 2017). One of the most common procedures that reduce the amount of alien chromosome present is the induction of crop-alien Robertsonian translocations (RobTs). This approach requires a set of specific genetic stock plants, including MAALs or MASLs as a donor, and monosomic plants with a single acceptor chromosome (Kwiatk et al., 2017b).

The main aim of this study was to generate the monosomic addition and substitution line of triticale with introgression of alien $2S^k$ chromosome derived from *A. kotschy*, which could be used for efficient study and transfer of genes responsible for leaf and stripe rust resistance.

MATERIALS AND METHODS

Plant Material

An artificial amphidiploid line of *Aegilops kotschy* × *Secale cereale* ($2n = 6x = 42$; $U^kU^kS^kS^kRR$) (**Figure 1**) was obtained by Wojciechowska and Pudelska (2002) from a cross between *A. kotschy* Boiss. (no. 14,808; kindly provided from Prof. Taihachi Kawahara, Kyoto University, Kyoto, Japan; **Figures 2a,b**), and diploid rye “Dankowskie Złote” (Danko Hodowla Roślin sp. z o.o.). Triticale “Sekundo” (Danko Hodowla Roślin Sp. z o.o.) was used as a pollinator for F_1 seed production and five subsequent backcrosses (**Figure 3**). Nullisomic (N2R) line of triticale were developed by Kwiatek et al. (2016c) and used for crossing with monosomic alien addition of $2S^k$ chromosome to produce monosomic $2S^k(2R)$ substitution plants of triticale (**Figure 3**). Cross-hybridization was made in greenhouse of the Institute of Plant Genetics of the Polish Academy of Sciences. Anthers of maternal plants were emasculated and spikes isolated using paper bags, in order to avoid uncontrolled pollination. Mature stigmas were pollinated with the pollen of triticale “Sekundo.” Crossing efficiency percentage was calculated by dividing of the total amount of seeds with total number of pollinated flowers.

Cytogenetic Studies

Accumulation of cells at mitotic metaphase and fixation was carried out according to Kwiatek et al. (2017b). Root meristems were digested at 37°C for 2 h and 40 min in an enzymes solution containing 0.2% (v/v) Cellulase Onozuka R-10 and Calbiochem cytohelase (1:1 ratio) and 20% pectinase (Sigma), in 10 mM citrate buffer (pH 4.6). Digested root tips were placed on slides with a drop of ice-cold 60% acetic acid and squashed. The coverslips were removed with a razor after liquid nitrogen treatment.

Probe Preparation and Fluorescence *in situ* Hybridization

Total genomic DNA was purified using GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Gdańsk, Poland). DNA of *Aegilops sharonensis* Eig (a progenitor of the S^k -genome of *A. kotschy*; PI 551020, U.S. National Plant Germplasm System) and *A. umbellulata* (a progenitor of the U^k -genome of *A. kotschy*; PI 298905, U.S. National Plant Germplasm System) were labeled by nick translation with Atto-488NT and Atto-550NT kits (Jena Bioscience, Jena, Germany), respectively. Blocking DNA from triticale “Sekundo” was prepared by boiling for 30–45 min (1:50 probe: block ratio). Chromosomes were identified using fluorescence *in situ* hybridization (FISH) with the repetitive sequences from pTa-86, pTa-535, pTa-103 (centromere specific), and pTa-k374 (homologous to ribosomal DNA sequence 28S) clones described by Komuro et al. (2013). Clones were amplified from genomic DNA of “Chinese Spring” wheat (Kwiatek et al., 2016b; Goriewa-Duba et al., 2018) and labeled using Atto-488NT, Atto-550NT, and Atto-647NT nick translation kits (Jena Bioscience, Germany). FISH/genomic *in situ* hybridization (GISH) experiments were performed

according to Kwiatek et al. (2017b). Slides were examined with the Olympus BX 61 automatic epifluorescence microscope equipped with Olympus XM10 CCD camera. Image processing was performed using Olympus Cell-F (version 3.1; Olympus Soft Imaging Solutions GmbH; Münster, Germany). Chromosomes of *Aegilops* and triticale were identified by comparing the signal patterns of the probes (Badaeva et al., 2004; Komuro et al., 2013; Kwiatek et al., 2013; Ruban and Badaeva, 2018).

SSR Marker Analysis

The SSR marker S14-410 (forward primer: 5'-ACCAATTCAACTTGCCAAGAG-3'; reverse primer: 5'-GAGTAACATGCAGAAAACGACA-3; Smit, 2013) closely linked to the *Lr54 + Yr37* loci on the $2S^k$ chromosome was used to genotype genomic DNA of the plant materials. Genomic DNA of *Aegilops kotschy*, triticale “Sekundo” and the hybrid plants were isolated using Plant DNA Purification Kit (EurX Ltd., Gdańsk, Poland). PCR reactions were performed in a LabCycler thermal cycler (SensoQuest Biomedizinische Elektronik, Goettingen, Germany). The PCR reaction consisted of 150 nM each primer (Merck KGaA, Darmstadt, Germany), 0.2 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.2 units of Taq-DNA hot-start polymerase (TaqNovaHS, Blirt, Gdańsk, Poland), and 50 ng of genomic DNA as a template. The PCR conditions were as follows: 5 min at 95°C, then 35 cycles of 30 s at 94°C, 30 s at 61°C, 1 min at 72°C, and 5 min at 72°C. Midori Green Direct (Nippon Genetics Europe) was added to each amplification product and analyzed by gel electrophoresis on 2% agarose gel (LabEmpire, Poznań, Poland). Gels were visualized and photographed using EZ GelDoc System (BioRad, Hercules, CA, United States).

Evaluation of Leaf Rust Symptoms in Growth Chamber

Evaluation of the response of monosomic substitution plants on leaf rust infection was conducted in the growth chamber (at IPG PAS) using leaf and stripe rust urediniospores, which were collected separately from triticale fields in three localizations in Wielkopolska region: IPG PAS Experimental Station in Cerekwica, Poland (52° 31' 16" N, 16° 41' 30" E); Experimental Station of the Poznań University of Life Sciences (PULS), Dłonów, Poland (51°41'22"N 17°04'23"E); and Experimental Garden of the Department of Genetics and Plant Breeding (PULS) in Poznań (52° 25' 26" N, 16° 54' 07" E). The leaf and stripe rust symptoms were scored in two independent and separate experiments (**Figure 4**). Both experiments were repeated. Each experiment repeat included forty monosomic $2S^k(2R)$ substitution plants and forty control plants (“Sekundo”), which were sprayed with leaf rust urediniospore solution containing 0.1% Tween 20, at three-leaf stage. Another 160 plants were sprayed with stripe rust urediniospore solution. The inoculated plants were then incubated in a humid growth chamber free from light for 10 days. After inoculation, the plants were maintained under a day/night photoperiod of 18/6 h, a temperature of 16–22°C. Winter triticale cv. “Sekundo” was taken as the susceptible control. The infection type of each individual was scored at three timepoints [10, 15, and 20 days post-inoculation (*dpi*)] using an

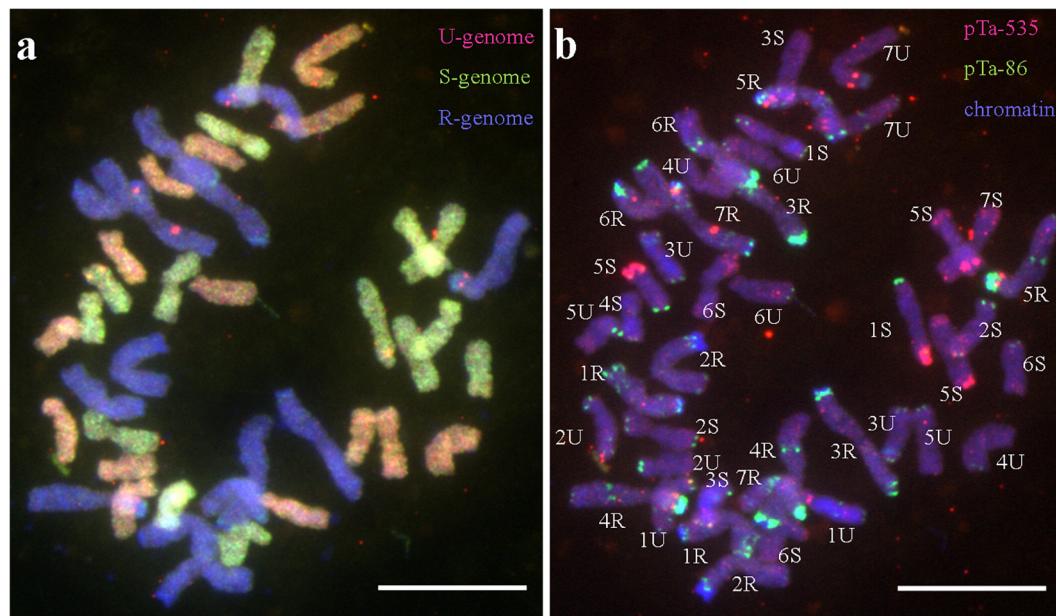


FIGURE 1 | Fluorescence/genomic *in situ* hybridization performed on mitotic chromosomes of *A. kotschy* × *S. cereale* amphiploid. **(a)** U^k - and S^k -genome chromosomes are labeled with Atto-550 (red) and Atto-488 (green), respectively. R-genome chromosomes labeled with DAPI (blue). **(b)** Probes pTa-535 and pTa-86 are labeled with Atto-550 (red) and Atto-488 (green), respectively. Chromosomes counterstained with DAPI (blue). Scale bar, 10 μ m.

infection scale adapted from Roelfs (1988) and transformed into nine-grade scale (1, high resistance; 9, susceptibility, **Tables 2, 3**; McNeal et al., 1971). The phenotypic data were analyzed using analysis of variance (ANOVA) and Tukey's highest significant difference (HSD) test.

RESULTS

Production of Monosomic $2S^k$ Addition Plants From Triticale-*A. kotschy* Hybrids

Seven F_1 plants were produced from a cross between hexaploid triticale "Sekundo" ($2n = 6x = 42$ chromosomes; AABBRR) and hexaploid *A. kotschy* × *S. cereale* ($2n = 6x = 42$; UUSSRR; **Figure 2c**) hybrid, as a pollen donor. All seven F_1 plants possessed 42 chromosomes including a complete haploid set of chromosomes of *A. kotschy* (**Figure 5a** and **Table 1**). The number of R-genome chromosomes was 14. Fifteen BC_1F_1 seeds were obtained from 375 flowers of F_1 plants, which were backcrossed using triticale pollen. FISH experiments revealed that chromosomes $1U^k$, $2U^k$, and $7S^k$ were eliminated (**Figure 5b** and **Table 1**). Fiftyone BC_2F_1 seeds were obtained by subsequent backcrossing of BC_1F_1 with triticale "Sekundo." Fifty BC_2F_1 plants were karyotyped (**Table 1**), with the progressive elimination of *Aegilops* chromosomes was observed. The number of U-genome chromosomes varied between 1 and 2. The number of S^k -genome chromosomes was much more diversified and varied between 2 and 6 (**Figure 5c** and **Table 1**). However, two chromosomes ($2S^k$ and $4S^k$) were present in all 50 BC_2F_1 plants (**Table 1**).

The BC_2F_1 plants were pollinated with triticale "Sekundo" pollen, and a total number of 234 seeds were obtained. FISH was carried out on 100 BC_3F_1 , revealing that chromosome $7U^k$ was present in all plants. Chromosome number of S^k -genome varied between 2 and 3 (**Figure 5d**). As before, chromosomes $2S^k$ and $4S^k$ were present in all 100 BC_3F_1 plants (**Table 1**). Fifty randomly selected plants of 93 BC_4F_1 hybrids were karyotyped using FISH and GISH techniques. Chromosomes of U^k -genome were completely eliminated, but surprisingly chromosomes $2S^k$ and $4S^k$ were present in all 50 BC_4F_1 plants (**Figure 5e** and **Table 1**). The last backcross resulted in formation of 216 BC_5F_1 seeds (**Table 1**). One hundred plants were karyotyped and 99 of them carried additional $2S^k$ chromosomes ($2n = 43$) (**Figure 5f** and **Table 1**). Chromosome $2S^k$ was not present in one BC_5F_1 plant ($2n = 42$) (**Table 1**), and this plant showed centric breaks in three pairs chromosomes (**Figure 6**). Therefore, an investigation of six generations of triticale Sekundo × (*A. kotschy* × *S. cereale*) hybrids (F_1 to BC_5F_1) by means of molecular cytogenetic methods (FISH and GISH) revealed subsequent elimination of *Aegilops* chromosomes.

Production of Monosomic $2S^k(2R)$ Substitution Plants From Triticale-*A. kotschy* Hybrids

A total number of 468 seeds were obtained from a cross between nullisomic N2R triticale plants ($2n = 40$; **Figures 7a,b**) and monosomic $2S^k$ addition line (MA $2S^k$ AL) of triticale (**Figures 7c,d**), as a pollen donor. 6,486 flowers of N2R plants were emasculated and pollinated with MA $2S^k$ pollen. The

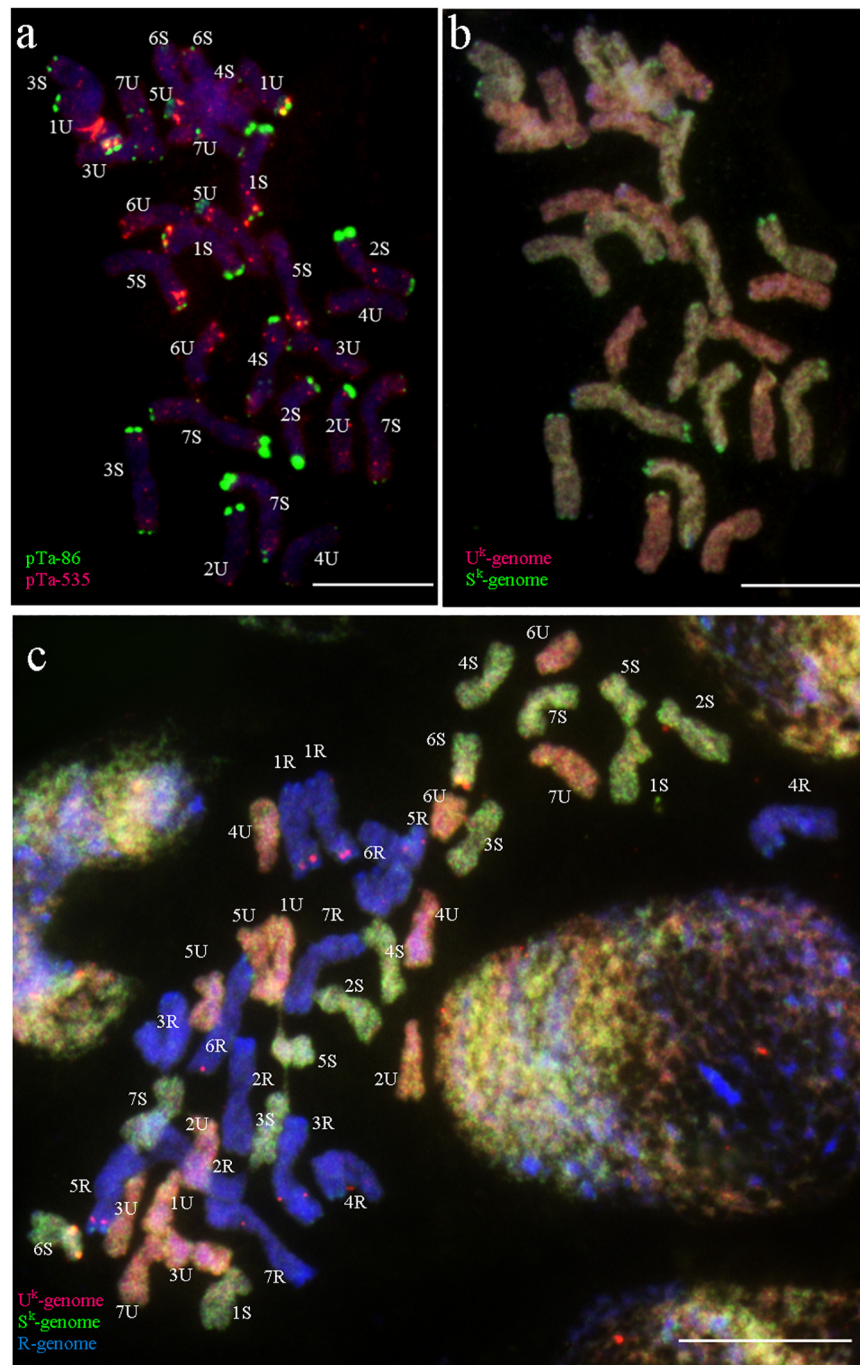


FIGURE 2 | Fluorescence/genomic *in situ* hybridization performed on mitotic chromosomes of *A. kotschy* Boiss ($2n = 6x = 42$; $U^kU^kS^kS^k$; no. 14808 using (a) pTa-86 (Atto-488, green), pTa-535 (Atto-550, red). Genomic *in situ* hybridization using U-genome (Atto-550, red) and S-genome (Atto-488, green) probes performed on mitotic chromosomes of (b) *A. kotschy* and (c) *Aegilops kotschy* \times *Secale cereale* ($2n = 6x = 42$; UUMMR). (a,c) Chromosomes counterstained with DAPI (blue). Scale bar, 10 μ m.

crossing efficiency was 7.22%, and 468 plants were obtained. The FISH/GISH analyses revealed that each offspring plant carried 42 chromosomes, including 2R and 2S^k chromosomes, which were in monosomic condition (Figure 7e,f). The presence of monosomic or nullisomic 2R chromosome was not observed.

Lr54 + Yr37 SSR Markers Analysis

PCR with SSR marker S14-410, linked to *Lr54* + *Yr37* loci, was conducted on *A. kotschy*, triticale “Sekundo,” the monosomic substitution M2S^k (M2R) line and nullisomic N2R triticale plants ($2n = 40$). The same protocol was used to examine

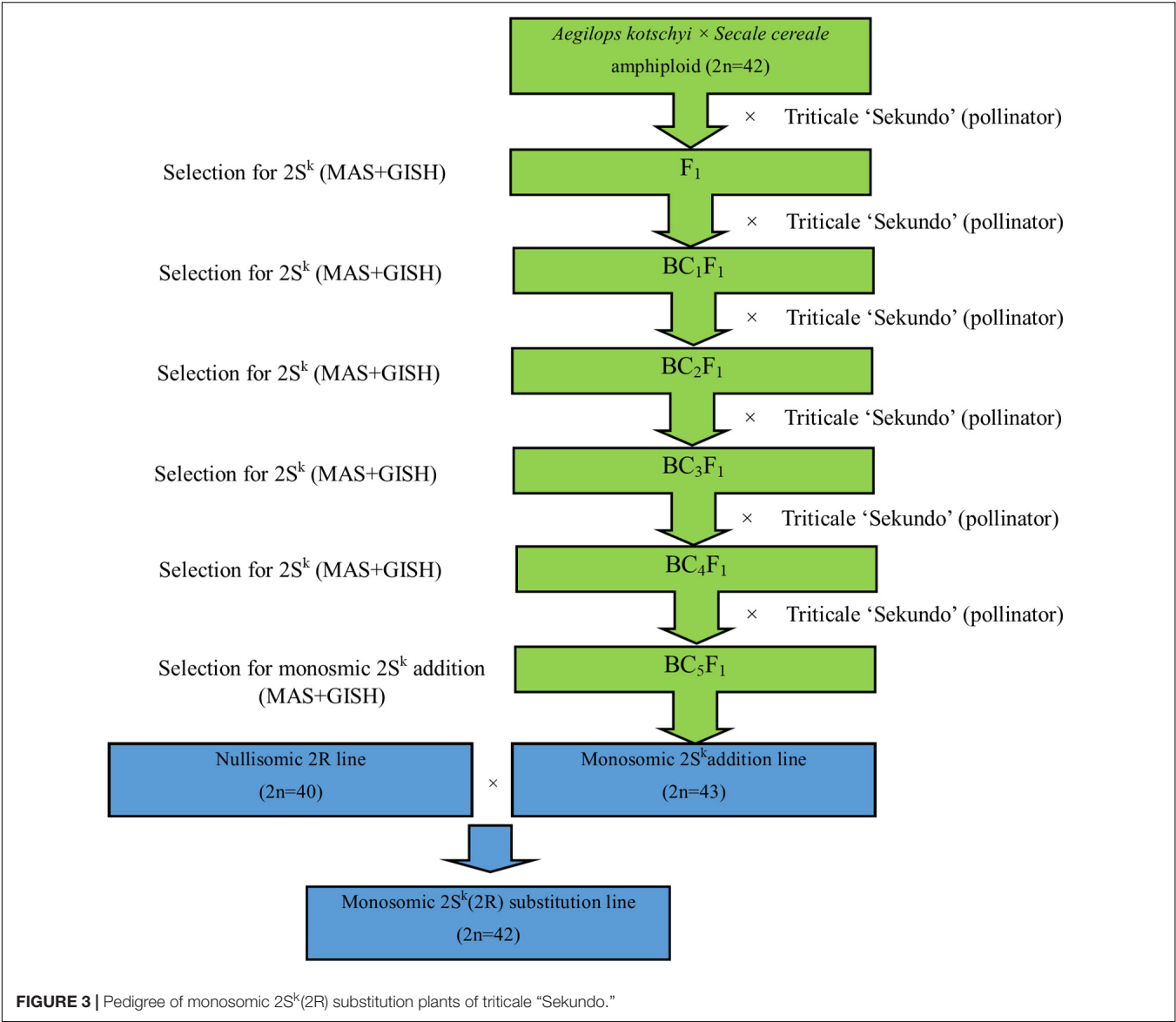


TABLE 1 | Frequencies of individual *Aegilops kotschy* chromosomes in five subsequent generations of backcrossing with triticale.

| Generation | Pedegree | Number of pollinated flowers | Number of seeds | Number of plants (FISH) | Number of plants with | | | | | | | | | | | | | |
|--------------------------------|--|------------------------------------|--------------------|----------------------------|-----------------------|---|----|----|----|----|-----|----------------------|-----|----|-----|----|----|---|
| | | | | | U-genome chromosomes | | | | | | | S-genome chromosomes | | | | | | |
| | | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| F ₁ | Sekundo × AkSc* | 710 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | |
| BC ₁ F ₁ | F ₁ × Sekundo | 375 | 15 | 15 | 0 | 0 | 13 | 2 | 11 | 13 | 15 | 1 | 15 | 12 | 15 | 5 | 1 | 0 |
| BC ₂ F ₁ | BC ₁ F ₁ × Sekundo | 488 | 51 | 50 | 0 | 0 | 0 | 42 | 0 | 0 | 50 | 5 | 50 | 41 | 50 | 23 | 36 | 0 |
| BC ₃ F ₁ | BC ₂ F ₁ × Sekundo | 715 | 234 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 23 | 0 |
| BC ₄ F ₁ | BC ₃ F ₁ × Sekundo | 480 | 94 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 50 | 0 | 0 | 0 |
| BC ₅ F ₁ | BC ₄ F ₁ × Sekundo | 487 | 216 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 99 | 0 | 0 | 0 | 0 | 0 |

*AkSc, *Aegilops kotschy* × *Secale cereale* amphiploid (2n = 6x = 42 chromosomes, U^kU^kS^kS^kRR).

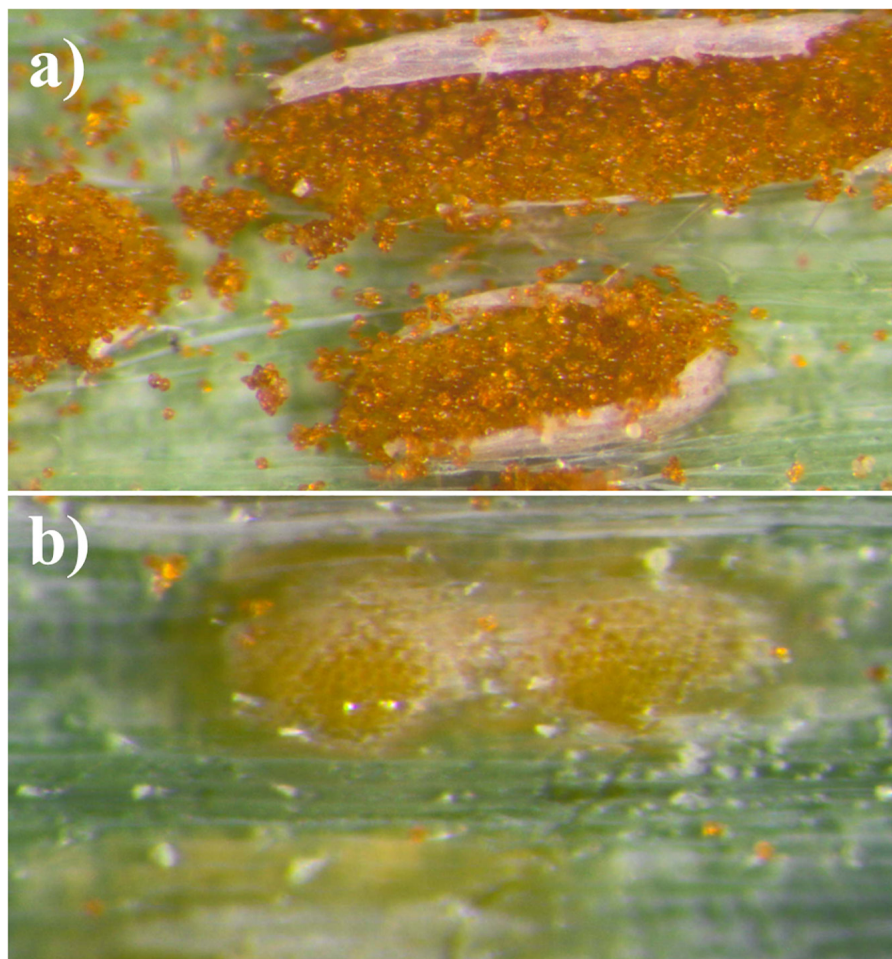


FIGURE 4 | Symptoms of **(a)** leaf rust and **(b)** stripe rust observed on leaves of triticale “Sekundo.”

TABLE 2 | Means of leaf rust infection levels scored 10, 15, and 20 days post-inoculation (dpi).

| Plant material | Experiment | No of plants tested | Means of infection levels | | | |
|--|------------|---------------------|---------------------------|--------|--------|-----------------|
| | | | 10 dpi | 15 dpi | 20 dpi | Mean \pm SD |
| Monosomic 2S ^k (2R) substitution plants | 1 | 40 | 2.60 | 3.00 | 3.30 | 2.97 \pm 0.48 |
| Triticale cv. “Sekundo” | 1 | 40 | 5.90 | 6.43 | 6.88 | 6.40 \pm 0.73 |
| Monosomic 2S ^k (2R) substitution plants | 2 | 40 | 2.70 | 2.98 | 3.20 | 2.96 \pm 0.42 |
| Triticale cv. “Sekundo” | 2 | 40 | 5.86 | 6.45 | 6.95 | 6.43 \pm 0.75 |

F ratio = 1,267.62 (*Supplementary Table 1*).

TABLE 3 | Means of stripe rust infection levels scored 10, 15, and 20 days post-inoculation (dpi).

| Plant material | Experiment | No of plants tested | Means of infection levels | | | |
|--|------------|---------------------|---------------------------|--------|--------|-----------------|
| | | | 10 dpi | 15 dpi | 20 dpi | Mean \pm SD |
| Monosomic 2S ^k (2R) substitution plants | 1 | 40 | 2.85 | 3.18 | 3.85 | 3.29 \pm 0.77 |
| Triticale cv. “Sekundo” | 1 | 40 | 2.73 | 3.20 | 3.80 | 3.24 \pm 0.79 |
| Monosomic 2S ^k (2R) substitution plants | 2 | 40 | 2.70 | 3.15 | 3.78 | 3.21 \pm 0.79 |
| Triticale cv. “Sekundo” | 2 | 40 | 2.67 | 3.30 | 4.00 | 3.33 \pm 0.85 |

F ratio = 0.5 (*Supplementary Table 2*).

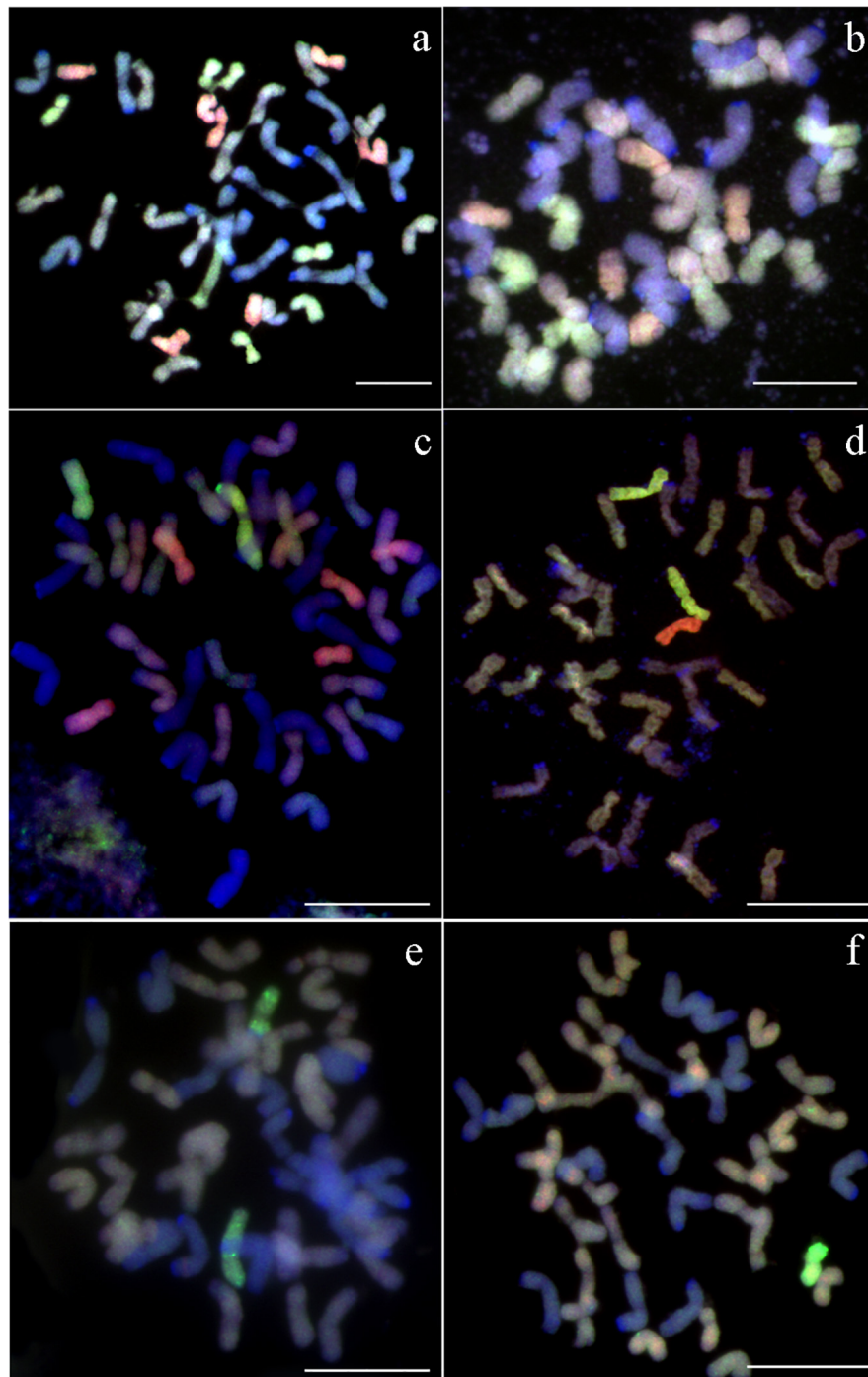


FIGURE 5 | Genomic *in situ* hybridization performed on mitotic chromosomes of (a) F₁, (b) BC₁F₁, (c) BC₂F₁, (d) BC₃F₁, (e) BC₄F₁, and (f) BC₅F₁ triticale-*Aegilops* introgression plants. A- and B-genome chromosomes are labeled with DAPI (light blue), as well as R-genome chromosomes (dark blue). U^k- and S^k-genome chromosomes are labeled with Atto-550 (red) and Atto-488 (green), respectively. Scale bar, 10 μm.

the offspring derived by subsequent backcrossing. A 410-bp amplicon for the S14-410 marker was identified in the control sample of *A. kotschy* and in all hybrid plants of F₁ to BC₄F₁ generations (**Figure 8**). Screening for the presence of the S14-410 marker in 100 BC₅F₁ plants showed that 410 bp amplicon

was present in 99 plants. Similar screening was carried out in 468 plants, created by cross-hybridization of N2R triticale plants ($2n = 40$) and M2S^kAL plants ($2n = 43$). PCR reaction with the genomic DNA of all recombinant plants yielded a 410-bp fragment.

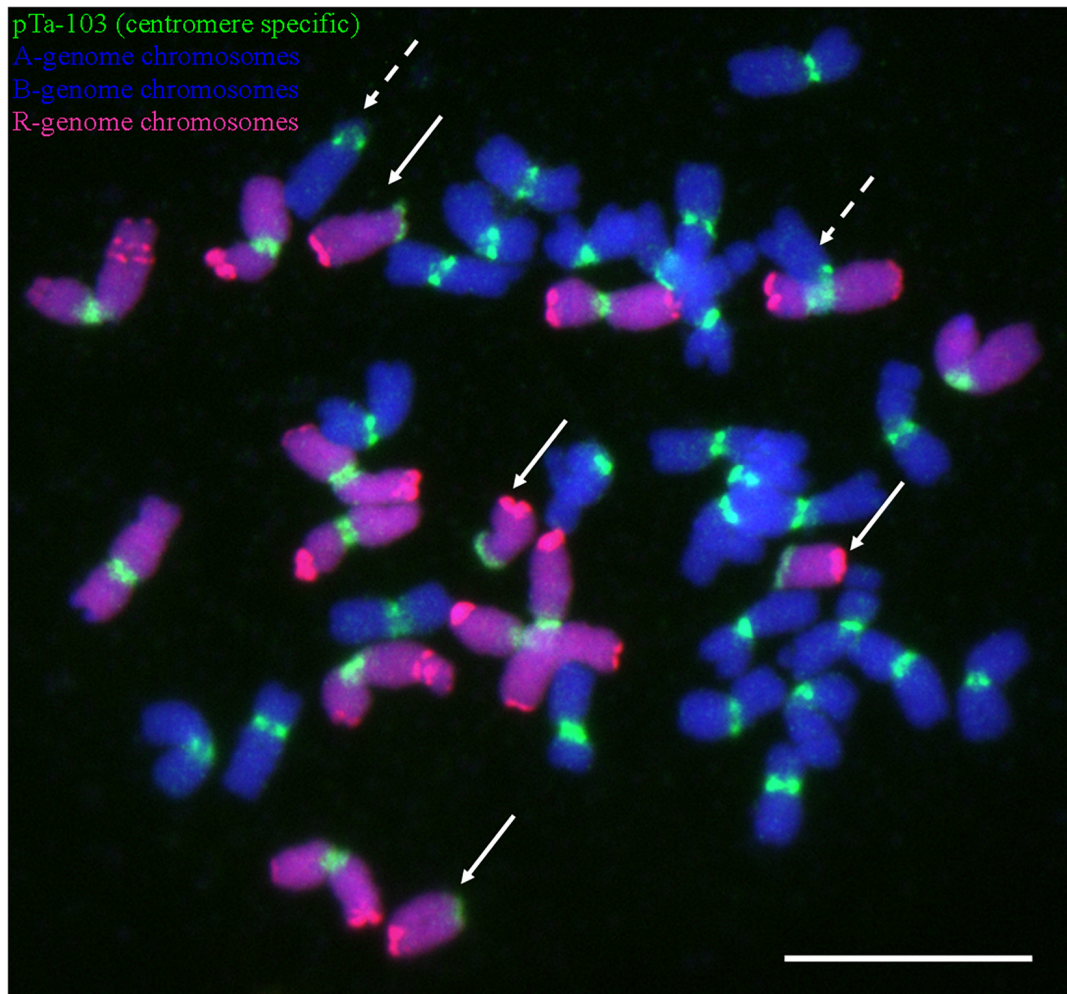


FIGURE 6 | Centric breaks of chromosomes in BC_5F_1 plant screened using multi-color GISH/FISH. Centromere regions were mapped using pTa-103 (Atto-488, green) probe. Dotted arrows, chromosome aberrations of A- or B-genome (DAPI, blue) of triticale; solid arrows, chromosome aberrations of R-genome (Atto-550, red) of triticale. Scale bar, 10 μ m.

Evaluation of Leaf Rust Symptoms

The leaf and stripe rust response of two sets of 80 plants of triticale monosomic $2S^k(2R)$ substitution line was evaluated at the seedling stage in the growth chamber and compared with phenotypes of acceptor cultivar “Sekundo” as controls (Table 2). The mean scores for two replications of control plants (6.40 ± 0.73 and 6.43 ± 0.75) showed that the inoculation solution was effective for induction of the leaf rust infection. The mean score of three independent evaluations of infection level (10, 15, and 20 dpi) of two replications of monosomic $2S^k(2R)$ substitution plants was similar (2.96 ± 0.42 and 2.97 ± 0.48) (Table 2). The differences were significant at $\alpha = 0.01$ (Supplementary Table 1). The scores for two replications of the leaf rust response experiment did not differ significantly (Supplementary Table 1). In contrast, the stripe rust responses of the monosomic $2S^k(2R)$ substitution plants and the control triticale cv. “Sekundo” at the three successive points did not differ significantly ($\alpha = 0.01$) (Table 3). Plants of cv. “Sekundo” showed

moderate stripe rust resistance (3.24 ± 0.79 and 3.33 ± 0.85), as well as monosomic substitution plants (3.21 ± 0.79 and 3.29 ± 0.77). ANOVA test for all three timepoints showed that there is no statistically significant differences between stripe rust response of monosomic substitution plants compared with triticale “Sekundo” controls (Supplementary Table 2).

DISCUSSION

Alien and cultivated species are closely related and possess homeologous genomes (or sub-genomes). Hence, it is possible to introduce the desirable variation into crop plants using a backcrossing program. However, isolation barriers exist that prevent the formation of interspecific hybridization, and present a significant obstacle for the exploration of alien genetic variation in crop improvement. The generation of artificial amphidiploids can facilitate the transmission of desirable genetic

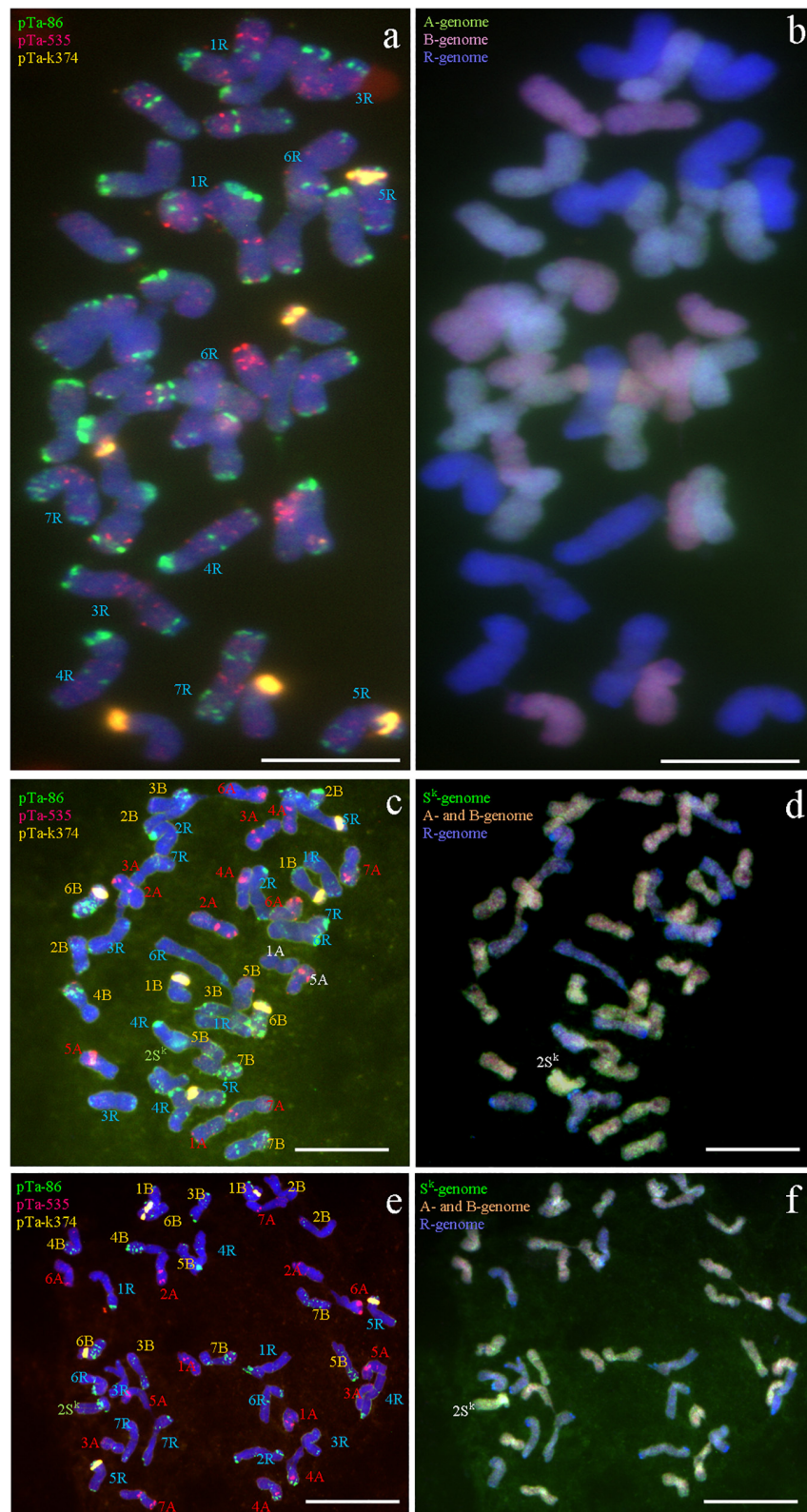


FIGURE 7 | Fluorescence and genomic *in situ* hybridization performed on mitotic chromosomes of: **(a,b)** nullisomic 2R plant of triticale ($2n = 40$); **(c,d)** monosomic 2S^k addition plant of triticale ($2n = 43$); **(e,f)** monosomic 2S^k(2R) substitution plant of triticale ($2n = 42$). pTa-86 (Atto-488; green), pTa-535 (Atto-550; red), and pTa-k374 (Atto-647; yellow) probes were used for FISH. Genomic probes of A-, B-, R-, and S^k-genome were used for GISH. Scale bar, 10 μm.

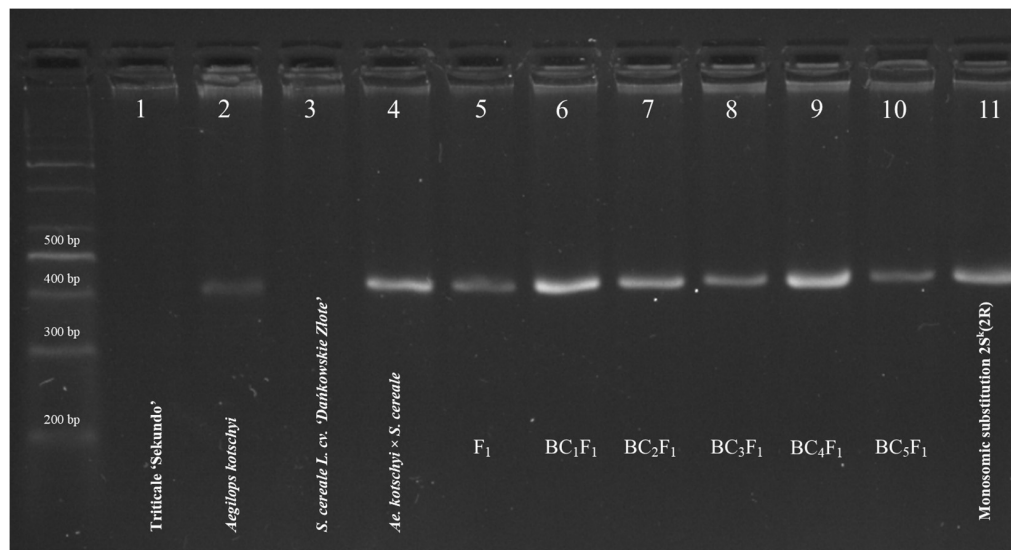


FIGURE 8 | Amplification products (410 bp) of S14-410 marker linked to loci of *Lr54* + *Lr37* leaf and stem rust resistance genes on 2S^k chromosome of *Ae. kotschy*. (1) triticale “Sekundo” (AABBRR); (2) *Aegilops kotschy* (U^kU^kS^kS^k); (3) *Secale cereale* L. “Dańkowskie Złote”; (4) *Aegilops kotschy* × *Secale cereale* (2n = 6x = 42; UUMRRR); (5) F₁; (6) BC₁F₁; (7) BC₂F₁; (8) BC₃F₁; (9) BC₄F₁; (10) BC₅F₁; and (11) monosomic 2S^k(2R) substitution plant of triticale (2n = 42). Size standard: Thermo Scientific™ GeneRuler™ 100 bp DNA Ladder.

material from wild species to crops. In this study, synthetic *A. kotschy* × *S. cereale* (2n = 6x = 42, U^kU^kS^kS^kRR) amphidiploid plants were used as a donor of wild genetic material to widen the genetic variation in hexaploid triticale “Sekundo” (2n = 6x = 42, AABBRR).

Similar artificial amphidiploid forms were used to transfer *Lr32* and *Lr39* leaf rust resistance genes from *A. tauschii* × *S. cereale* (2n = 4x = 28; DRRR) (Kwiatk et al., 2015) and *Pm13* powdery mildew resistance gene from *A. variabilis* × *S. cereale* (2n = 6x = 42, U^vU^vS^vS^vRR) (Kwiatk et al., 2016a) into cultivated triticale. It was reported that the presence of an R-genome chromosome set resulted in semi-fertile F₁ plants that were capable of producing an F₁ via cross-hybridization of artificial *Aegilops biuncialis* × *S. cereale* (2n = 6x = 42, U^bU^bM^bM^bRR) amphiploid and hexaploid triticale (Kwiatk et al., 2017a). In this study, seven F₁ plants were obtained via cross-hybridization of *A. kotschy* × *S. cereale* and triticale. The first backcross with triticale “Sekundo” resulted in rapid elimination of the *Aegilops* chromosomes. The elimination range of the U^k-genome chromosomes was far higher when compared with the rate of S^k-genome chromosome loss (Table 1). The elimination of alien chromosomes was also observed in the BC₂F₁ generation. Interestingly, only 7U^k, 4U^k, 2S^k, and 4S^k chromosomes were present in all of BC₂F₁ plants. In subsequent generations of triticale hybrids, obtained via successive backcrosses, the number of *Aegilops* chromosomes was highly reduced and the plants of BC₄F₁ carried 2S^k and 4S^k chromosomes only.

This pattern could be a result of gametocidal action of the *Gc* locus on 2S^{sh} of *Aegilops sharonensis* (Endo, 1985). Moreover, similar “breaker” element described in *A. sharonensis* (Maan, 1975). These loci have been mapped to

a region proximal to a block of sub-telomeric heterochromatin on chromosome 4S^{sh}L (Knight et al., 2015). The diploid *A. sharonensis* (2n = 2x = 14; S^{sh}S^{sh}) is a direct ancestor and a donor of S^k-genome of *A. kotschy* (Kihara, 1954; Badaeva et al., 2004; Ruban and Badaeva, 2018). Investigations into the origin of *A. kotschy* showed that the U^k and S^k subgenomes are very similar to the diploid progenitors: *A. umbellulata* (2n = 2x = 14; UU) and *A. sharonensis*, respectively (Zohary and Feldman, 1962; Zohary, 1999; Ozkan et al., 2001; Feldman and Levy, 2012). It is a distinct possibility that there are gametocidal loci on 2S^k and 4S^k chromosomes of *A. kotschy*, and those loci could be orthologous to *A. sharonensis* analogs.

In the presented study, 99% of BC₅F₁ monosomic alien addition plants of triticale carried an additional 2S^k chromosome. Endo (2007) suggested, that when the gametocidal action is intense, gametes without the alien chromosome may suffer severe chromosome damages, resulting in sterility. In this study, one BC₅F₁ plant without 2S^k chromosomes suffered minor chromosome aberrations (Figure 3). It could be hypothesized that gametes without 2S^k can be fertile and develop into plants with chromosome aberrations as no chromosome fragment has been lost. This genetic phenomenon is of particular interest to breeders. Linking *Gc* loci with *Lr54* + *Yr37* leaf and stripe rust-resistant genes in triticale varieties would ensure maintenance of these traits in subsequent generations without the need for selection. But, on the other hand, triticale plants with centric chromosome breaks can be selected and used for the induction of Robertsonian translocations (RobTs) (Kwiatk and Nawracała, 2018).

The molecular analyses with the S14-410 marker confirmed that the preferential transmission of the 2S^k chromosome

occurred in all hybrid plants, from the F_1 generation to BC_4F_1 . The *Lr54* + *Yr17* marker identification reflected the results of cytogenetic analysis of BC_5F_1 plants. The key result of this study was the development of monosomic $2S^k(2R)$ substitution lines, which can be used for induction of chromosome translocations. There are several reports describing the methodology used for the production of monosomic substitutions. According to Faris et al. (2002), this can be achieved by crossing an alien chromosome addition line containing the gene of interest with a line that is monosomic for a homoeologous wheat chromosome. This approach was applied for development of $1RS.1DL$ chromosome translocation in triticale “Rhino” (Lukaszewski and Curtis, 2006). The $1RS.1DL$ translocation was later used for the induction of multi-breakpoint translocation lines (Lukaszewski, 2006, 2017).

It is reported that triticale is infected by the races specific to both: wheat and rye; however, it was noticed that triticale is more easily attacked by the wheat physiological forms of the rusts than by the rye ones (Arseniuk, 1996). After inoculation, a significant improvement of the resistance level was observed in introgressed plants in comparison with triticale cv. Sekundo plants. The mean level of leaf rust resistance was high for two independent replications of the experiment (2.96 ± 0.42 and 2.97 ± 0.48). Such low infection rate can be considered as a result of *Lr54* gene expression. In a similar study, Marais et al. (2005) developed a $2DS.2S^kL$ wheat-*A. kotschy* line (called S14 translocation). The authors reported 96% resistant plants (72 tested) which were tested for resistance to eight *Pt* pathotypes (UVPrt2, UVPrt3, UVPrt4, UVPrt5, UVPrt8, UVPrt9, UVPrt10 and UVPrt13) and two *Pst* pathotypes (6E16A- and 6E22A-) endemic to South Africa. Moreover, it was shown that the S14 translocation evidently had preferential transmission (Marais et al., 2005). Intriguingly, monosomic $2S^k(2R)$ substitution triticale plants, as well as control plants revealed the same, moderate resistance against stripe rust. In general, it is reported that triticale is more resistant against stripe rust as wheat. Moreover, triticale infection by *P. striiformis* is highly dependent on plant growth stage and pathogen race (Rodriguez-Algaba et al., 2020). Hence, it could be possible that triticale “Sekundo” possesses a partial resistance against stripe rust and *Yr37* did not increase it.

In this study, the monosomic $2S^k(2R)$ substitution plants were obtained through crossing between monosomic $2S^k$ addition line of triticale ($M2S^kAL$), containing *Lr54* loci, with another triticale line that was nullisomic for 2R chromosome pair ($N2R$). The cross-hybridization between $M2S^kAL$ and $N2R$ plants resulted in no offspring with the 2R chromosome in a monosomic condition ($M2R$). Hence, it can be hypothesized that gametocidal action of $2S^k$ chromosome killed gametes lacking it. Moreover, it could be possible that the inhibiting or suppressing factor of the $2S^k$ gametocidal action in triticale is located on the 2R chromosome. It is already known that some cultivars of common wheat possess genes that suppress the function of the *Gc* factors. Tsujimoto and Tsunewaki (1985) reported that a suppressor gene (*Igc1*), that controls the suppression of *Gc* gene action on chromosome 3C of *A. truncialis*, is located on 3B chromosome of wheat “Norin 26.” Similarly, Endo (1988) postulated that gametocidal action of the 4S chromosome of

A. sharonensis or *A. longissima* is suppressed by the presence of 4B chromosome of wheat. It could be plausible that both the *Gc* gene and the suppressor loci are located on chromosomes of the same homoeologous group; however, these hypotheses require future investigation.

The classical methods basing on backcrossing and selection are still significant in plant breeding. There are several countries, which are considered as key crop producers (for example, France, Germany, Poland, etc.), place a strict set of regulations on the cultivation of genetically modified (GM) crops. Hence, classical breeding methods, enriched by the molecular (and cytomolecular)-assisted selection and chromosome manipulation are still worthy endeavors. In addition, the genetic mechanisms that regulate the process of segregation distortion should be seriously taken into consideration as a natural tool for chromosome manipulation and engineering.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MK: conceptualization, methodology, formal analysis, writing original draft preparation, project administration, and funding acquisition. MK, WU, JB, and HW: validation. MK, JB, WU, RS, and AN: investigation. MK and HW: resources and supervision. MK and WU: data curation. MK and DP: writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.509481/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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